Modified Polyelectrolyte Microcapsules as Smart Defense Systems

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A new type of protective microcontainer, capable of preventing oxidation of encapsulated compounds by low molecular weight oxidizing agents, was demonstrated. These microcontainers (microcapsules) are hollow spheres with the shell made of layer-by-layer assembled polyelectrolytes with an engineered outermost layer. The protecting ability of the developed polyelectrolyte capsules was monitored by oxidation of encapsulated bovine serum albumin by H_2O_2 dissolved in aqueous solution. Different catalysts for H_2O_2 decomposition (catalase, Fe_3O_4 nanoparticles) were deposited onto the polyelectrolyte shell to increase the protection efficiency. The highest protection activity was observed for poly(allylamine)/poly(styrene sulfonate) capsules containing nano-Fe_3O_4 in the polyelectrolyte shell.

Many natural and artificial objects are subjected to degradation by the surrounding environment. It is especially important to protect compounds that can be damaged by irradiation, heating, oxidizing (reducing) agents, polymers, moisture, etc., on the molecular level during their storage and targeted delivery. Such substances are of practical importance in medicine (drugs), gene engineering (DNA, RNA), biotechnology (proteins, individual cells, enzymes), and the food industry. Development of protective microenvironments combining defense property with the others, for instance the possibility of controlled release of the secured compound at the desired site in aggressive media, is of particular interest. Two general methodological approaches for protection are isolation of the substance inside a defensive microvolume or modification of the substance (saving desired properties) with formulations or functional groups more sensitive to a certain type of aggressive treatment (such as oxidation, hydrolysis, etc.) than the protected substance itself. The latter approach is well-developed and there are a number of publications describing the coupling of the substance to be protected (DNA, metal nanoparticles, quantum dots, etc.) with proteins,[1] poly(amines),[2] metal ions,[3] amino acids,[4] surfactants,[5] antioxidants,[6] aromatic sulfonates,[7] and chelate agents.[8] Embedding substances inside a microvolume was shown using lipid-based microenvironments,[9] liposomes,[10] microbeads,[11] (bio)polymer capsules,[12] amalgam capsules,[13] emulsions and vesicles,[14] and gels[15] as protective microcontainers. However, the significant shortcoming of the protective microcontainers developed so far is their monofunctionality, i.e., the protection ability is not superposed with the other properties such as luminescent or magnetic functions.

Recently developed layer-by-layer (LbL) technology[16] offers the opportunity to fabricate multifunctional nanoengineered microcontainers. This technology is based on sequential deposition of oppositely charged polyelectrolytes on a surface of variable shape, which allows the formation of multilayer shells from a wide range of the components with nanometer precision.

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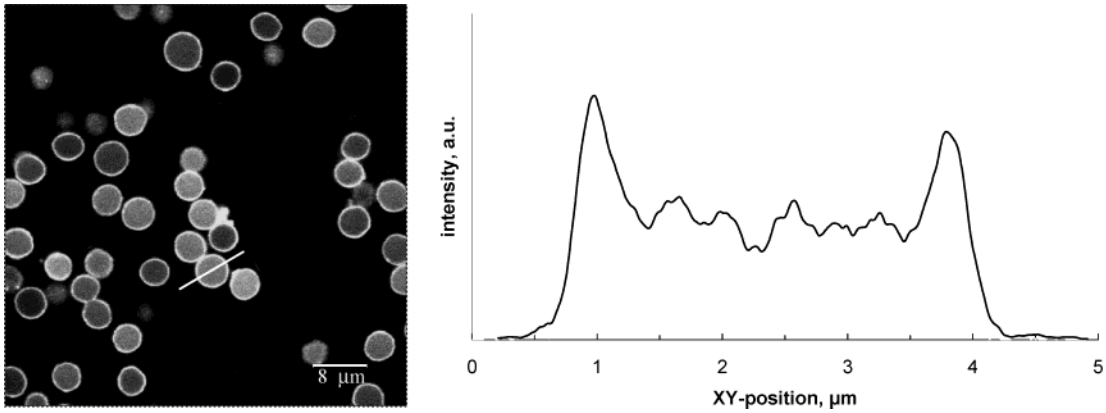

Figure 1. Confocal fluorescence microscopy image and fluorescence profile of BSA-loaded $(PAH/PSS)_4-PAH$ polyelectrolyte capsules. Encapsulated BSA molecules were labeled with fluorescein isothiocyanate.

Templating of LbL polyelectrolyte films on the surface of the micron- and submicron-sized isolated particles followed by their dissolution leads to formation of hollow polyelectrolyte capsules.[17] The initial colloidal core determines the size of the capsules, which can be varied from 50 μm to 50 nm. The polyelectrolyte shell is permeable for small molecules and ions,[18] while large molecules and nanoparticles can be entrapped inside or banned from the capsule interior. Sustained release of the encapsulated furocemide, dextran, and fluorescein molecules was demonstrated.[19] Different additional functionalities (magnetic, luminescent, sensor) can be imparted to the capsule shell introducing nano-Fe_3O_4, q-CdS, etc. as shell constituent.[20]

Due to the semipermeability of the polyelectrolyte shell, polyelectrolyte capsules can act as microcontainers, effectively protecting encapsulated material against large polymer molecules by excluding them in outer solution[19a] and, at the same time, maintaining controlled release of encapsulated material. In situ modification of the capsule shell with inorganic nanoparticles lends mechanical stability (nano-YF_3, nano-hydroxyapatite) and UV-protection ability (nano-TiO_2) to the polyelectrolyte shell.

Here, we adjust properties of LbL-assembled polyelectrolyte capsules toward protection of encapsulated material against low molecular weight oxidative species. H_2O_2, capable to be catalytically decomposed into H_2O and O_2, was taken as the model oxidant. Bovine serum albumin (BSA) was employed as a model protein to be protected against oxidation in the capsule interior, due to the extensive characterization of its properties in reaction with peroxo radicals generated from both H_2O_2 and other peroxides.[21]

Results and Discussion

The main products of the interaction between BSA and H_2O_2 are carbonyl moieties, which can be analyzed by reliable assays. A confocal fluorescence image of polyelectrolyte capsules containing labeled FITC−BSA, which was used to control the encapsulation procedure, is presented in Figure 1. As a result, approximately 90% of the capsules are filled with BSA homogeneously distributed in the capsule volume (see fluorescence profile for FITC−BSA in Figure 1). The small excess at the capsule wall is caused by electrostatic interactions between BSA and the charged groups of the polyelectrolytes. It is important to emphasize that BSA used in further experiments was not labeled. The quantity of BSA, measured using the Lowry method,[23] is 2.5 pg per capsule.

Three types of modified polyelectrolyte capsules were tested for protection against H_2O_2 oxidation of encapsulated BSA. As first example, $(PAH/PSS)_4-PAH$ microcapsules with PAH as the outer layer were investigated. The presence of polyelectrolytes in the shell anticipates the antioxidant properties of the shell, because they can be irreversibly oxidized by H_2O_2, thus forming a sacrificial barrier for H_2O_2 going through the shell inside the capsule. Two other kinds of protective microcapsules are $(PAH/PSS)_4-PAH$ capsules surface-engineered by depositing on the outer layer a catalyst for H_2O_2 decomposition to H_2O and less active O_2. Nanosized Fe_3O_4 and the enzyme catalase were chosen as catalysts for shell modification.

Fe_3O_4 nanoparticles were introduced in the polyelectrolyte shell before BSA encapsulation by either adsorption of preformed Fe_3O_4 from magnetic fluid or by in situ synthesis of nano-Fe_3O_4 directly in the shell from a solution of 0.1 M $FeSO_4$ + 0.1 M $Fe_2(SO_4)_3$ according to the procedure described in ref 25. The presence of nanosized Fe_3O_4 is seen in the TEM image of ultrami-

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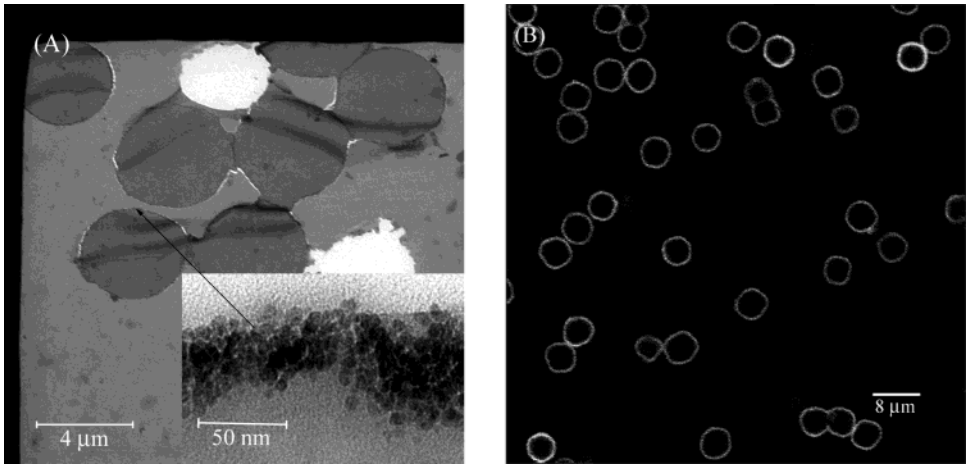

Figure 2. TEM image of Fe_3O_4-containing polyelectrolyte capsules (a) and confocal fluorescence image of $(PAH/PSS)_4-PAH$ capsules with a deposited outmost catalase layer. Catalase molecules were labeled with fluorescein isothiocyanate.

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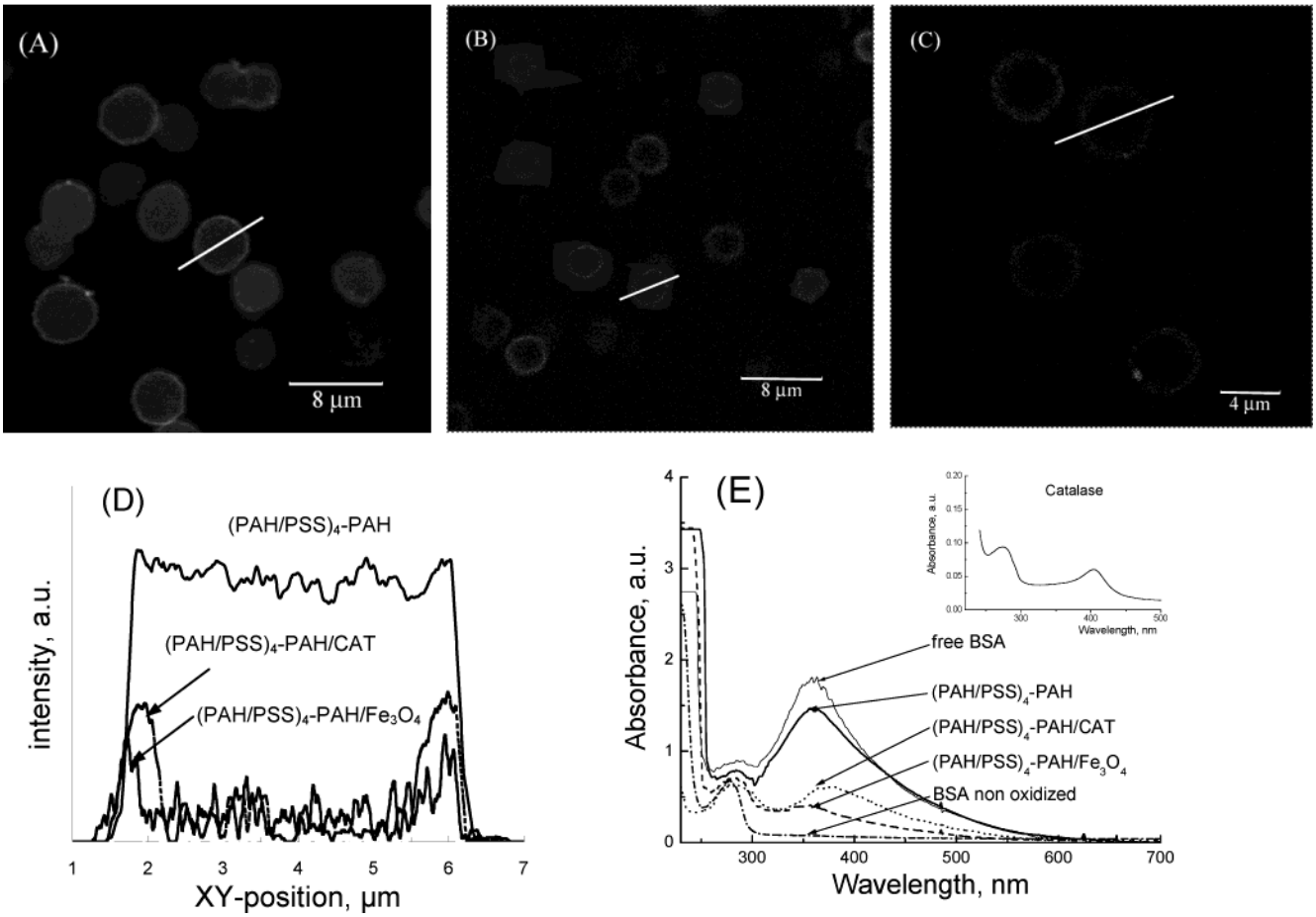

Figure 3. Oxidation of BSA encapsulated in $(PAH/PSS)_4-PAH$ (a), $(PAH/PSS)_4-PAH$/catalase (b), and $(PAH/PSS)_4-PAH/Fe_3O_4$ (c) microcapsules. Carbonyl moieties of BSA were visualized by the reaction with fluorescein isothiocyanate labeled carbazide. (d) Fluorescence profiles for a single capsule. (e) UV/vis spectra of encapsulated BSA derivatized with 2,4-dinitrophenylhydrazine after H_2O_2 treatment. Soret band of the catalase heme occurring in the blue region has negligible influence on the spectrum of BSA derivatized with 2,4-dinitrophenylhydrazine (see catalase absorption on the inset in Figure 3e).

crotomed polyelectrolyte capsules (Figure 2a). Dense arrangement of Fe_3O_4 nanoparticles in the polyelectrolyte shell was observed and no magnetite precipitate was detected in the capsule volume. Formation of magnetite nanoparticles during in situ synthesis in the capsule shell was confirmed by wide-angle X-ray scattering. The average size of the Fe_3O_4 nanoparticles is 4 nm. The mass increase of a single polyelectrolyte capsule after Fe_3O_4 deposition, measured by quartz crystal microbalance technique, is ~61wt %.

In the other case, the deposition of catalase on the surface of positively charged $(PAH/PSS)_4-PAH$ capsules was performed at a pH value above the isoelectric point of the enzyme (~5.4) to attain catalase molecules with

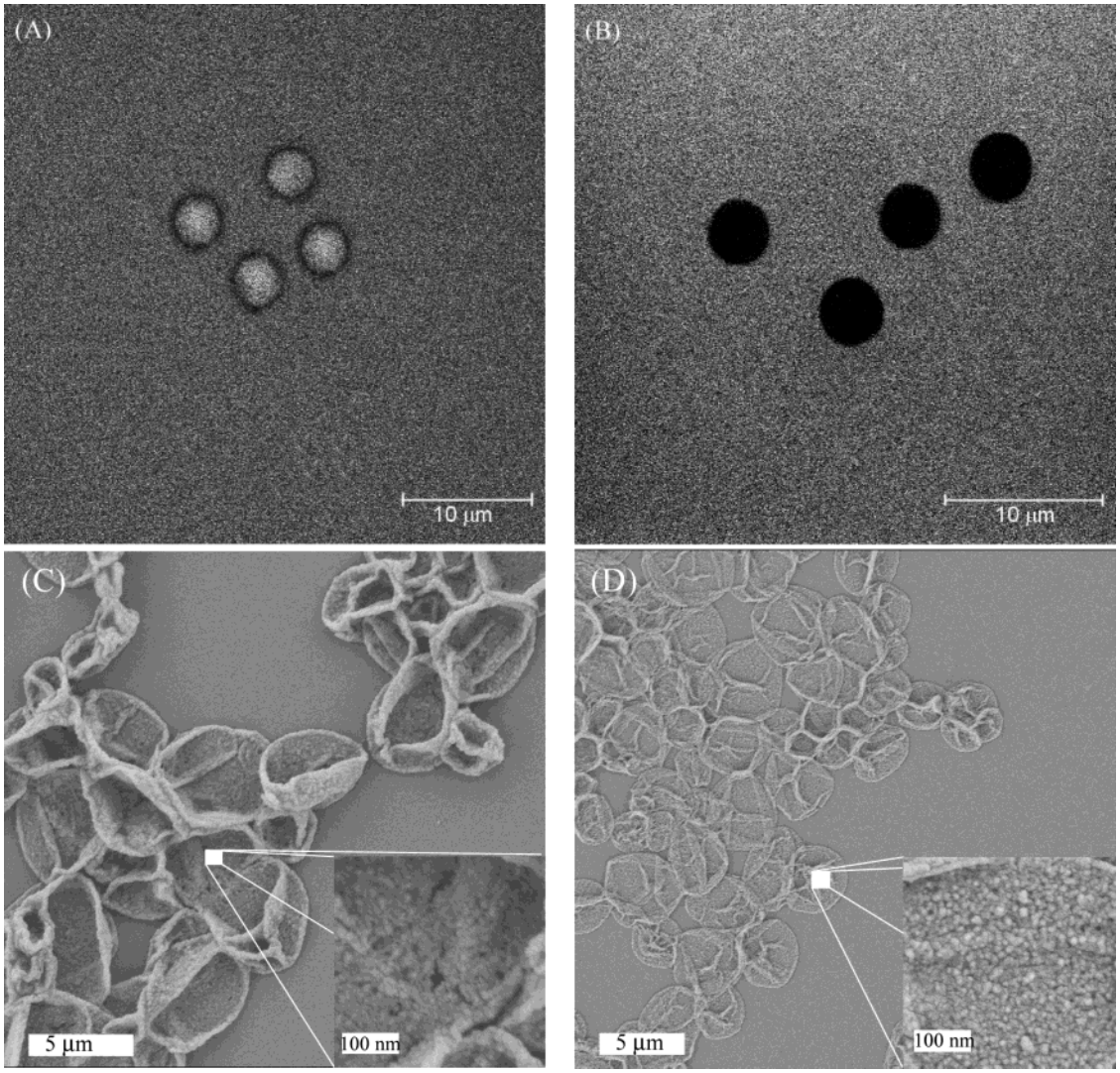

Figure 4. Confocal fluorescent microscopy images of $(PAH/PSS)_4-PAH$ (a) and $(PAH/PSS)_4-PAH/Fe_3O_4$ (b) microcapsules in the solution containing fluorescein-labeled dextran (MW = 2000 kDa) at pH = 9. Microcapsules were treated in 0.5 M H_2O_2 solution before measurements. The changes in the shell permeability are clearly seen. Scanning electron microscopy images of $(PAH/PSS)_4-PAH$ (c) and $(PAH/PSS)_4-PAH/CAT$ (d) microcapsules after treatment in 0.5 M H_2O_2. The differences of the shell morphology are revealed in the insets for SEM images.

a negative charge. The outer catalase layer was formed from 2.5 mg/mL CAT solution at pH = 8 with 3 h. of incubation. Then, the rest of undeposited catalase was washed out with deionized water. Visualization of the catalase layer on the surface of the polyelectrolyte capsules was made using labeled FITC−CAT. As seen in Figure 2b, a homogeneous catalase layer was obtained. The thickness of the catalase layer deposited on the PAH is ca. 2 nm (according to quartz crystal microbalance measurements). The mass increase of a single polyelectrolyte capsule after catalase deposition is ~18 wt %

Polyelectrolyte capsules loaded with unlabeled BSA were added to 0.1 M solution of H_2O_2 for 30 min. For comparison, an equivalent quantity of free BSA was also exposed to H_2O_2. The H_2O_2 solution was activated by a small amount of aqueous ammonia. The quantity of the resulting carbonyl groups was measured by their derivatization with 2,4-dinitrophenylhydrazine and visualized by confocal fluorescence microscopy upon reaction with FITC-carbazide.

The initial $(PAH/PSS)_4-PAH$ shell reveals a small protection effect caused by the partial reduction of H_2O_2 molecules that penetrate the polyelectrolyte shell (Figure 3). Sacrificial oxidation of the shell material (PAH, PSS) leads to the loss of integrity, controlled permeability properties, and, as a consequence, to the destruction of the polyelectrolyte shell at high H_2O_2 concentrations or prolonged H_2O_2 treatment (Figure 4). $(PAH/PSS)_4-PAH$ polyelectrolyte capsules become permeable for fluorescein-labeled dextran of MW = 2000 kDa at alkali pH = 9 after treatment in 0.5 M H_2O_2 (Figure 4a), while initial $(PAH/PSS)_4-PAH$ capsules (before oxidation) are closed for dextran molecules of 2000 kDa at alkali pH.[18] Formation of cracks and holes in the polyelectrolyte shell was also observed in 0.5 M H_2O_2 solution (Figure 4c). The protection efficiency can be increased by doping the polyelectrolyte shell with an active electron donor (e.g. poly(vinyl alcohol)[26]); however, the limited protection capacity, described above, still remains.

One of the possible approaches to eliminate this drawback is to create an "active" barrier on the surface

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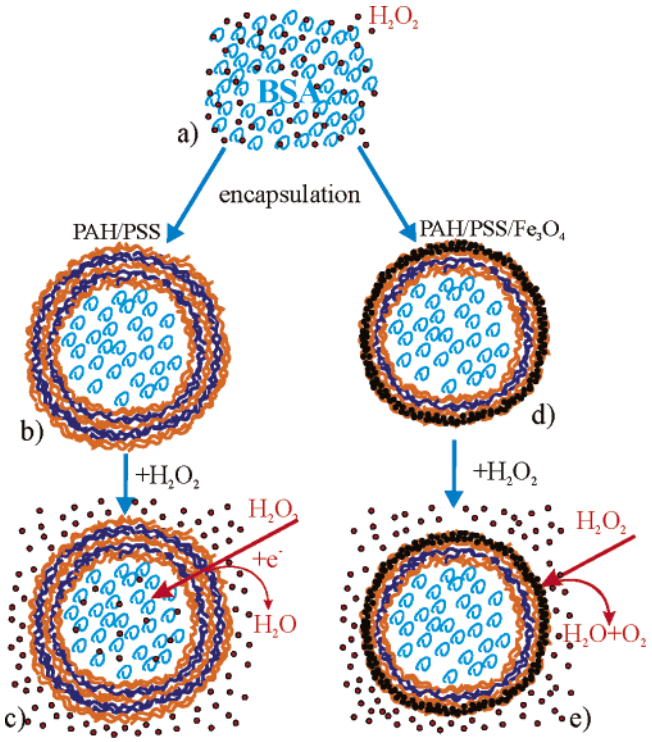


Figure 5. Schematic overview of the "passive" and "active" protection approaches for nanoengineered polyelectrolyte capsules.

of the $(PAH/PSS)_4$−PAH microcapsule, i.e., to combine a passive redox mechanism with an active catalytic protection. Modification of the polyelectrolyte shell with nano-Fe_3O_4 or CAT, which catalyzes H_2O_2 decomposition to O_2, results in a drastic increase of the antioxidant properties of the shell and decreases the number of carbonyl groups in encapsulated BSA after H_2O_2 treatment (Figure 3b−e). The most effective is a nano-Fe_3O_4 layer; a similar protection yield was observed for both preformed and in situ synthesized Fe_3O_4. The catalase-modified polyelectrolyte capsules are 1.5 times less effective as compared to Fe_3O_4-modified ones, possibly due to partial deactivation of the enzyme after adsorption on PAH. Addition of the active outer layer increases the stability and integrity of the capsule shell (Figure 4d). They preserve their permeability properties for high molecular weight molecules remaining in the "close" state for dextran of 2000 kDa at pH = 9 (Figure 4b).

Both types of protective mechanisms are schematically represented in Figure 5. For "passive" shells, part of the H_2O_2 is reduced by the PAH/PSS shell material. "Active" ones combine catalytic and redox mechanisms to provide a more efficient protection barrier. The magnetic activity of polyelectrolyte capsules doped with Fe_3O_4 is also preserved after (and during) H_2O_2 treatment.

In the presented work, we developed protective polyelectrolyte microcapsules that effectively prevent the oxidation of encapsulated bovine serum albumin by a low molecular weight agent, H_2O_2. Two approaches for designing protective capsule microcontainers are demonstrated: The "passive armor" approach is composed of a sacrificial reducing agent as a shell constituent, while the "active armor" approach includes the catalyst for H_2O_2 decomposition deposited onto the shell as the outer layer. In the latter case, the protective material is not consumed during the H_2O_2 treatment, thus prolonging the protection activity of the microcapsule. The designed microcontainers combine the protective function with the controlled release of the encapsulated substance and, moreover, can possess magnetic activity to perform magnetically adjusted delivery of the encapsulated material in aggressive media. Protective polyelectrolyte capsules can find applications as delivery and depot systems in medicine, drug industry, and biotechnology. The possibility of using developed microcontainers for preserving the bioactivity of different encapsulated enzymes (urease, chymotripsin) in oxidative media is under investigation now.

Experimental Section

Materials. Sodium poly(styrene sulfonate) (PSS, MW ~ 70 000), poly(allylamine hydrochloride) (PAH, MW ~ 50 000), H_2O_2, bovine serum albumin (BSA), HCl, NaOH, fluorescein-labeled bovine serum albumin (FITC−BSA), fluorescein-labeled carbazide (FITC-carbazide), fluorescein-labeled catalase (FITC−CAT), NH_3, 2,4-dinitrophenylhydrazine (DNPH), catalase (CAT), trichloroacetic acid (TCA), $Fe_2(SO_4)_3 \cdot 7H_2O$, and $FeSO_4 \cdot 7H_2O$ were purchased from Sigma-Aldrich. Magnetic Fe_3O_4 was purchased from Berlin Heart AG. Monodispersed $MnCO_3$ template particles with a diameter of 3.8 μm were synthesized as described in ref 22.

All chemicals were used as received. The water was prepared in a three-stage Millipore Milli-Q Plus 185 purification system and had a resistivity higher than 18 MΩ·cm.

Fabrication of Polyelectrolyte Capsules. Hollow PAH/PSS capsules were prepared by following a previously published procedure[17] by alternating adsorption of oppositely charged PAH and PSS molecules from corresponding 2 mg/mL aqueous solutions on the surface of template $MnCO_3$ particles starting with a PAH layer. The resulting polyelectrolyte shell consists of four PAH/PSS bilayers and an outer layer of PAH $\{(PAH/PSS)_4-PAH\}$. The $MnCO_3$ core was dissolved in HCl (0.1 M) after formation of PAH/PSS shells.

Encapsulation of Bovine Serum Albumin. BSA was encapsulated by changing the permeability of the capsule shell at acidic and neutral pH.[19c] Polyelectrolyte capsules were added to an acidic solution (pH = 2, adjusted by HCl) containing BSA (10 mg/mL) and kept there for 24 h. Then, the pH was increased to 7 (by NaOH) and BSA-loaded capsules were washed out with deionized water. Confocal spectroscopy confirmed that the enzyme remains in the capsules after loading for at least 2 months. The concentration of BSA encapsulated inside the microcapsules was estimated by Lowry analysis of proteins.[23]

Characterization. *Confocal Microscopy.* Confocal fluorescence microscopy images of polyelectrolyte capsules in solution were obtained on a Leica TCS SP scanning system equipped with a 100× oil immersion objective and operating in fluorescein mode.

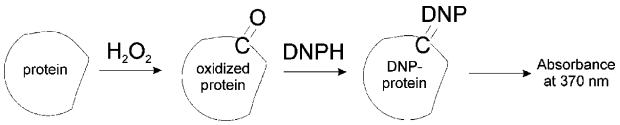
Scanning Electron Microscopy. The capsule suspension was dropped onto a glass wafer with sequential drying at room temperature overnight. Then the samples were sputtered with gold and measurements were conducted using a Gemini Leo 1550 instrument operated at an acceleration voltage of 3 keV.

Transmission Electron Microscopy. To view the interior structure, dried polyelectrolyte capsules were embedded into gelatin holders filled with polymerized MMA (methyl methacrylate) and then cut into ultrathin sections (from 30 to 100 nm in thickness) using a Leica ultracut UCT ultramicrotome. Copper grids coated with carbon film were used to support the thin sections and a Zeiss EM 912 Omega transmission electron microscope operating at 300 kV was employed for analysis.

Analysis of Carbonyl Groups. The quantity of carbonyl groups is a valid marker to detect oxidative protein modification.[21] Thus, the oxidation of BSA was monitored by the presence of carbonyl groups resulting from the reaction

between protein and H_2O_2.[24] Two independent analytical methods were used to measure the quantity of carbonyl groups in oxidized BSA.

(i) Oxidatively modified proteins were determined by derivatization of protein-bound carbonyl moieties with 2,4-dinitrophenylhydrazine according to the method of Reznick and Packer.[24a] For this assay, BSA-containing capsules after H_2O_2 treatment were extensively washed, and the protein was derivatized with DNPH overnight at room temterature. Non-encapsulated BSA, oxidized in a blank experiment under the same conditions as the encapsulated one, was precipitated with trichloroacetic acid, extensively washed with ethanol/ethyl acetate (1:1 vol/vol), redissolved in water, and derivatized with DNPH. The resulting dinitrophenylhydrazones were determined spectrophotometrically at 370 nm using an Agilent 8453 spectrophotometer.



(ii) To visualize oxidized bovine serum albumin in the capsule using the confocal fluorescence microscope, FITC-labeled carbazide was utilized to label the carbonyl groups. BSA-loaded capsules after oxidation were kept in FITC-carbazide (0.05 mg/mL solution) for 4 h at room temperature and continuous stirring. After that, capsules were extensively washed with deionized water and studied by a Leica TCS SP confocal microscope operating in the FITC mode.

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